Immobilization of His-tagged recombinant xylanase from *Penicillium occitanis* on Nickel-chelate Eupergit C for increasing digestibility of poultry feed

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Recombinant xylanase 2 from *Penicillium occitanis* expressed with an His-tag in *Pichia pastoris*, termed PoXyn2, was immobilized on nickel-chelate Eupergit C by covalent coupling reaction with a high immobilization yield up to 93.49%. Characterization of the immobilized PoXyn2 was further evaluated. The optimum pH was not affected by immobilization, but the immobilized PoXyn2 exhibited more acidic and large optimum pH range (pH 2.0–4.0) than that of the free PoXyn2 (pH 3.0). The free PoXyn2 had an optimum temperature of 50 °C, whereas that of the immobilized enzyme was shifted to 65 °C. Immobilization increased both pH stability and thermostability when compared with the free enzyme. Thermodynamically, increase in enthalpy and free energy change after covalent immobilization could be credited to the enhanced stability. Immobilized xylanase could be reused for 10 consecutive cycles retaining 60% of its initial activity. It was found to be effective in releasing reducing sugar from poultry feed. Immobilization on Eupergit C is important due to its mechanical resistance at high pH and temperature. Hence, considerable stability and reusability of bound enzyme may be advantageous for its industrial application.

Introduction

Xylan is the most abundant component of hemicelluloses in angiosperm plant cell walls and is the second most abundant carbohydrate in nature. Xylanases (EC 3.2.1.8; endo- β-1,4-D-xylanase) are mainly responsible for the hydrolysis of xylan with β-1,4-xylanolytic linkages.[1] Xylanases have been the focus of research owing to their industrial potential in many fields. They are, for instance, useful in pulp and paper industries, particularly for the facilitative role they play in the bleaching of craft pulp in order to reduce the amount of chlorine required for target pulp brightness.[2] A recent and exciting application of endoxylanases is the production of xylo-oligosaccharides with huge commercial value, because xylo-oligosaccharides, especially xylobiose, have been found to have a stimulatory effect on the selective growth of human intestinal *Bifidobacteria*, and are frequently defined as prebiotics.[3,4] Another economically important application of Xyl-11 deals with animal feeding. For instance, in poultry production, xylanase supplementation in diet affects growth performance, digestion, and impact on immune parameters and gut microflora.[5] The utilization of thermostable enzymes might improve the technical and economic feasibility of all these biotechnological processes.

Because of the industrial potential of xylanases, a large number of studies have

become interested in their immobilization for industrial application. Several advantages are gained through this technique and include the possibility of enzyme reuse, the enhancement of thermal stability, simplifying the product purification process, providing opportunities for scaling-up, and allowing the development of processes based on different reactor configurations and the reduction of operating costs.[6,7]

Covalent immobilization is often favored for biocatalyst production because the bonds formed are more stable than formed by electrostatic or adsorption.[8,9] The epoxy supports can be used for covalent immobilization of enzymes and present several advantages; for instance, they are very stable during storage and they can increase the enzyme stability by preventing the enzyme from interactions with external interfaces (air, oxygen, immiscible organic solvents, etc.).[10,11] Eupergit C which consists of macroporous beads with a diameter of 100–250 μm made by copolymerization of N,N-methylen-bis-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether, and methacrylamide is very desirable for industrial scale enzyme immobilization because it is commercially available worldwide, resistant to mechanical and chemical stresses, and adaptable to a variety of configurations and specific processes performed in reactors.[12] Also, it may be very suitable to achieve the multipoint covalent attachment of enzymes, therefore, to stabilize their three-dimensional structure. Hence, it has been identified as the suitable carrier for covalent immobilization of enzymes for industrial applications.[8,12,13]

Many reports on the immobilization of mesophilic and moderately thermophilic xylanases have been published.[12-18]

The *Penicillium occitanis* Pol6 xylanase termed PoXyn2 was successfully expressed with an His-tag in the methylotrophic yeast *Pichia pastoris* X-33 under the control of the glyceraldehyde 3-phosphate dehydrogenase (GAP) constitutive promoter.[19]

Based on our recent work[20] the goal of this addendum is to study the effect of temperature and thermodynamics on the activity of free and immobilized enzyme

and to further exploit it in increasing digestibility of poultry feed.

Effect of Temperature on Immobilized Xylanase

To study the temperature dependence, activities of the free and immobilized xylanases were determined at different temperatures at pH 3.0.

The effect of temperature on the activity of soluble and immobilized xylanase was determined by carrying out the reaction at various temperatures ranging from 30 to 75 °C. The temperature showing maximum activity was taken as optimum for the enzyme. The residual activity (%) at each temperature was calculated by considering the enzyme activity at the optimum temperature as 100%. The activation energy (Ea) of catalysis for both the free and immobilized xylanase forms was determined from the slope of the Arrhenius plot (log V [logarithm of % residual activity] vs. reciprocal of absolute temperature in Kelvin [$1000/T$]), which is given by the following expression:

$$slope = -\frac{Ea}{R}$$

To study the thermal stability, both free and immobilized enzyme forms were pre-incubated at different temperatures ranging from 50 to 75 °C up to 2 h. Samples were withdrawn at 30 min intervals and analyzed for activity in standard enzyme assays at the optimum temperature. The residual activity was calculated by taking the enzyme activity at 0 min incubation as 100%. Results were also expressed as first order thermal deactivation rate constants (kd), half-lives ($t1/2$) and D-values (decimal reduction time or time required to pre-incubate the enzyme at a given temperature to maintain 10% residual activity) at each temperature. The kd was determined by regression plot of log relative activity (%) vs. time (min). The $t1/2$ and D-value of immobilized xylanase were determined from the relationships.

$$t\frac{1}{2} = \frac{\ln 2}{kd}$$

The temperature rise necessary to reduce D-value by one logarithmic cycle (z-value) was calculated from the slope of

graph between log D vs. T (°C) using the equation:

$$slope = -\frac{1}{Z}$$

The activation energy (Ed) for xylanase denaturation was determined by a plot of log denaturation rate constants (ln kd) vs. reciprocal of the absolute temperature (K) using the equation:

$$Slope = -\frac{Ed}{R}$$

The change in enthalpy ($\Delta H°$, kJ mol-1), free energy ($\Delta G°$, kJ mol-1) and entropy ($\Delta S°$, J mol-1 K-1) for thermal denaturation of xylanase were determined using the following equation:

$$\Delta H° = Ed - RT$$

$$\Delta G° = -RT \ln(kd \cdot \frac{h}{kB \cdot T})$$

$$\Delta S° = \frac{\Delta H° - \Delta G°}{T}$$

where T is the corresponding absolute temperature (K), R is the gas constant (8.314 J mol-1 K-1), h is the Planck constant (11.04 × 10^-36 J min), and k_B is the Boltzman constant (1.38 × 10^-23 J K-1).

Results showed that optimum temperature values of 50 and 60 °C were recorded for free and immobilized enzyme, respectively (**Fig. 1A**). A similar displacement of optimum temperature for immobilized enzymes has been observed in earlier studies but the extent of displacement varied from matrix to matrix and with the kind of interaction between enzyme and matrix.[21] The formation of intermolecular covalent bonds between the enzyme molecules and matrix support confer rigidity on the structure of the enzyme molecule, so that the enzyme is less affected by the denaturing effect of temperature.[22] The apparent activation energy (Ea) of catalysis for free and immobilized enzyme was calculated using Arrhenius plot (**Fig. 1B**). The regression equations for Arrhenius plots of free and immobilized xylanases were y = -2.145x + 8.787 and y = -1.058x + 4.984, respectively. The covalent immobilization of PoXyn2 lowered the Ea from 17.8 to 8.8 kJ mol-1, resulting in a higher catalytic efficiency of xylanase.

The thermal stability of the free and the immobilized PoXyn2 was studied in this work. It was seen that the free xylanase almost lost all its activity at 75 °C after 2 h
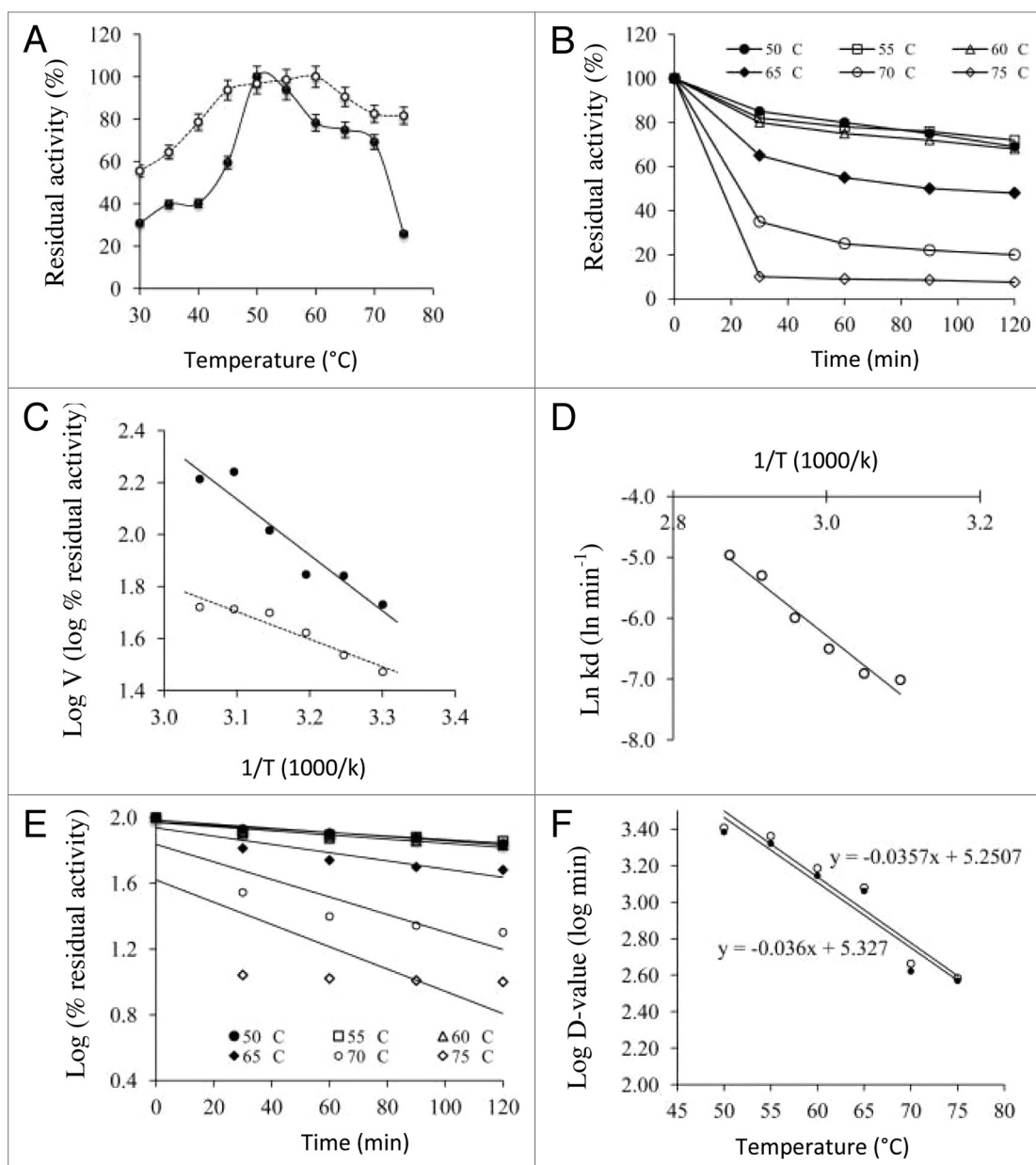
Figure 1. Effect of temperature on free (solid circle) and immobilized (hollow circle) xylanase: (**A**) temperature optima, (**B**) Arrhenius plot to calculate activation energy (E_a) of catalysis, (**C**) stability of immobilized xylanase, (**D**) first order thermal deactivation of the immobilized xylanase, (**E**) Arrhenius plot to calculate activation energy (E_d) for denaturation, and (**F**) temperature dependence of the decimal reduction of free and immobilized xylanase to calculate z-values.

of incubation (data not shown). However, xylanase immobilized on EupergitC preserved about 8% of its activity at 75 °C (**Fig. 1C**).

The increase in temperature optima and stability upon immobilization might be the result of enzyme rigidity.[23] Similar increase in thermo-stability of several enzymes on immobilization has been observed.[24] In general, the immobilization process of the enzyme was suggested to protect the enzyme against heat inactivation.[25] The restricted interaction between the immobilized enzyme molecules could also be responsible for retaining the enzyme activity at higher temperatures.[26] Enhanced stability of xylanase at higher temperature is likely to increase its suitability for industrial application.[27]

The rate of heat inactivation of immobilized enzyme was investigated in the temperature range between 50 °C and 75 °C. The plots of log (% remaining activity) vs. time were linear indicating the first order kinetics of immobilized enzyme (**Fig. 1D**). The thermostability parameters of free and immobilized xylanase are summarized in **Table 1**. The half-lives and D-values of PoXyn2 xylanase prolonged remarkably at all temperatures after covalent attachment indicating better thermostability of the immobilized xylanase. Tyagi and Gupta[28] also reported an increase in the half-life of Aspergillus xylanase at 60 °C on immobilization. To

Table 1. Kinetic and thermodynamic parameters for thermal inactivation of free and immobilized xylanase

		Temperature					
		50 °C	55 °C	60 °C	65 °C	70 °C	75 °C
k_d (min^{-1})	F [a]	0.00095	0.00110	0.00165	0.00200	0.00600	0.00650
	I [b]	0.00090	0.00100	0.00150	0.00190	0.00500	0.00600
$t_{1/2}$ (min)	F	729.60	630.10	420.00	346.50	115.50	106.60
	I	770.20	693.10	462.10	364.81	138.60	115.50
D-value (min)	F	2423.70	2093.20	1395.50	1151.30	383.70	354.20
	I	2558.40	2302.60	1535.10	1211.89	460.50	383.70
$\Delta H°$ (kJ mol^{-1})	F	73.94	73.90	73.86	73.82	73.78	73.74
	I	76.29	76.25	76.21	76.14	76.12	76.08
$\Delta G°$ (kJ mol^{-1})	F	109.00	110.33	110.93	112.10	110.92	112.23
	I	109.15	110.59	111.20	112.25	111.19	112.32
$\Delta S°$ (J mol^{-1} K^{-1})	F	-108.54	-111.07	-111.33	-113.26	-108.27	-110.61
	I	-101.73	-104.71	-105.08	-106.82	-102.23	-104.14

[a]Free enzyme. [b]Immobilized enzyme.

determine the thermodynamic parameters for thermal stability, the activation energy (Ed) for thermal denaturation was determined by applying the Arrhenius plot (**Fig. 1E**).

The Ed for immobilized enzyme was found 83.02 kJ mol^{-1}, which was higher in comparison to 79.27 kJ mol^{-1} of free enzyme indicating that immobilized enzyme was very stable, compact, and highly resistant to heat denaturation. The higher value of Ed meant more energy was required to denature the treated enzyme as postulated earlier.[29] A similar increase in thermostability of several enzymes on immobilization was observed by researchers.[30] At 50 °C, the ΔH° of free xylanase was 73.94 kJ mol^{-1} while that of immobilized enzyme was 76.29 kJ mol^{-1} which clearly indicated that more energy was required for thermal denaturation of the immobilized enzyme. The values of ΔH° decreased with increasing temperature, in both cases, revealing that lesser energy was required to denature enzyme at high temperatures but still more in immobilized xylanase (**Table 1**). The observed change in ΔH° also indicated that enzyme in both states exhibited a considerable conformational change at higher temperatures.[31]

The Gibbs free energy (ΔG°) of thermal unfolding apparently increased with increase in temperature but did not reveal large differences between free and immobilized enzyme forms. This indicated that the immobilization of PoXyn2 did not adversely affect its thermal unfolding at higher temperatures. The unfolding of enzyme structure was accompanied with an increase in disorder or entropy of deactivation, but xylanase had negative entropy (ΔS°) revealing that native form of enzyme was in more ordered state (**Table 1**). The immobilized enzyme also showed a negative value of ΔS° but the magnitude was substantially lesser as compared with free form. The less negative value for entropy in immobilized enzyme is normal due to the bonding.

Application of Immobilized Xylanase in Increasing Digestibility of Poultry Feed

The poultry feed was treated with optimized conditions of immobilized enzyme dose and treatment time to measure the reducing sugar content in feed. The poultry feed was collected from local market and re-suspended in water with a ratio of 1:2. The resulting suspension of feed was treated with varying amounts of immobilized PoXyn2 (5–20 units/g feed) for different time intervals (1–6 h). The immobilized xylanase were recovered from the feed by centrifugation at 2000 rpm for 1 min to obtain a clear supernatant for estimation of reducing sugars using 3,5-dinitrosalycyclic acid. The recovered enzyme from the feed were washed with distilled water and added to the next batch of the feed for treatment. **Figure 2A** showed that the maximum reducing sugars were obtained with 10 units of immobilized PoXyn2 after an incubation of 3 h. On increasing the dose of xylanase from 10 to 20 units there was no significant effect on reducing sugar contents of the feed. Similarly, on increasing the incubation time after 3 h there was a decline in reducing sugars. The exact reason for decline in reducing sugars is not clear but, it may be due to chemical changes in reducing sugars. The enzyme inactivation during prolonged treatment may be another factor responsible for decline in sugar content. Further, the feed was treated with optimized dose of bound enzyme and resulted in 82 mg reducing sugar per gram feed. The immobilized xylanase could be reused for 5 consecutive cycles for the treatment of feed (**Fig. 2B**). The recovery (%) of the immobilized xylanase after treating the poultry feed was 80.0 ± 5.0% at each cycle as compared with the preceding one. There is paucity of reports related to the enhancement in digestibility of poultry feed with immobilized xylanase. In fact, Maheshwari and Chandra[32] treated the starter feed with 5 units of xylanase, and
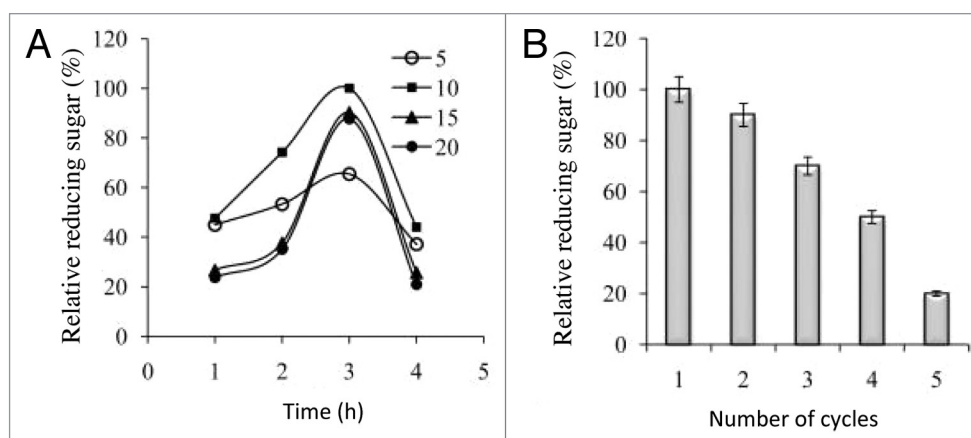
Figure 2. (**A**) The optimization of poultry feed at different enzyme doses (5–20 IU/g) with respect to time interval (1–4 h) in releasing reducing sugars. (**B**) Reusability of the immobilized xylanase in releasing reduced sugars from poultry feed at 50% consistency.

resulted in release of 21 mg/ml of reducing sugar.

Conclusion

Herein, the covalent immobilization of His tagged xylanase from *P.occitanis* lowered the activation energy, resulting in a higher catalytic efficiency of xylanase. The half-life time of the immobilized enzyme was longer compared with the free enzyme. In general, the evolution of the half-life time is regarded as an indicator for the efficiency of the immobilization process. Accordingly, the immobilized xylanase is suitable in the feed industries as is effective in releasing reducing sugars. This certainly prompts further investigations on the continuous, particularly the large-scale, production of reducing sugars.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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